Novel plasmid-based genetic tools for the study of promoters and terminators in *Streptococcus pneumoniae* and *Enterococcus faecalis*

Sofía Ruiz-Cruz, Virtu Solano-Collado, Manuel Espinosa and Alicia Bravo*

Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Ramiro de Maeztu, 9. E-28040 Madrid, Spain

* Corresponding author
Phone: (34) 918373112
Fax: (34) 915360432
E-mail: abravo@cib.csic.es

A B S T R A C T

Promoter-probe and terminator-probe plasmid vectors make possible to rapidly examine whether particular sequences function as promoter or terminator signals in various genetic backgrounds and under diverse environmental stimuli. At present, such plasmid-based genetic tools are very scarce in the Gram-positive pathogenic bacteria *Streptococcus pneumoniae* and *Enterococcus faecalis*. Hence, we developed novel promoter-probe and terminator-probe vectors based on the *Streptococcus agalactiae* pMV158 plasmid, which replicates autonomously in numerous Gram-positive bacteria. As reporter gene, a *gfp* allele encoding a variant of the green fluorescent protein was used. These genetic tools were shown to be suitable to assess the activity of promoters and terminators (both homologous and heterologous) in *S. pneumoniae* and *E. faecalis*. In addition, the promoter-probe vector was shown to be a valuable tool for the analysis of regulated promoters *in vivo*, such as the promoter of the pneumococcal fuculose kinase gene. These new plasmid vectors will be very useful for experimental verification of predicted promoter and terminator sequences, as well as for the construction of new inducible expression vectors. Given the promiscuity exhibited by the pMV158 replicon, these vectors could be used in a variety of Gram-positive bacteria.

Keywords: Gram-positive bacteria / Plasmids / pMV158 / Promoters / Terminators

1. Introduction

Identification of promoters and transcriptional terminators on the bacterial genomes is essential to understand the regulation of gene expression. In bacteria, numerous genes are organized in operons and, therefore, they are transcribed from the same promoter into a single polycistronic mRNA molecule. Moreover, many genes in known operons are transcribed from internal promoters, which are located at intergenic regions or within adjacent genes. Several highly accurate computational methods have been devised for detection of operons in bacterial genomes (for recent methods see Chuang et al., 2010; Taboada et al., 2010). As an example, operon predictions for 300 sequenced prokaryotic genomes are now available in the *Operons* database (http://operons.ibt.unam.mx/OperonPredictor/). Many algorithms have also been developed for the prediction of promoter sequences in genomic DNAs (Askary et al., 2009; Jacques et al., 2006). However, as pointed out by Ross and Gourse (2009), although bioinformatics can predict some promoters correctly, definitive identification of promoters requires the use of several experimental approaches, both *in vivo* and *in vitro*. These may include identification of the *in vivo* transcription start site using purified RNA, detection of promoter activity *in vivo* using promoter-reporter fusions and characterization of RNA polymerase-promoter complexes (*in vitro* transcription and DNA-binding assays).

The bacterial RNA polymerase (RNAP) holoenzyme is a complex of six subunits ($\alpha_2\beta\beta'\omega\sigma$). During initiation of transcription, most of the sequence-specific contacts of the RNAP with the promoter region are made by the σ subunit. In general, bacterial genomes encode diverse forms of the σ factor, and each of them confers promoter specificity to the RNAP (Gruber and Gross, 2003;

Wigneshweraraj et al., 2008). Most transcription in exponentially growing bacterial cells is initiated by RNAP carrying a housekeeping σ factor similar to the *Escherichia coli* σ^{70}. Promoters recognized by this holoenzyme are characterized by two main sequence elements, the –35 and –10 hexamers, whose consensus sequence is 5´-TTGACA-3´ and 5´-TATAAT-3´, respectively. The optimum spacer length between these elements is 17 nucleotides (for a review see Haugen et al., 2008). Additionally, some of these promoters contain the extended –10 element, which is located one nucleotide upstream of the –10 hexamer. This element is more conserved in Gram-positive bacteria (5´-TRTG-3´ motif) than in *E. coli* (5´-TG-3´ motif) (Mitchell et al., 2003; Sabelnikov et al., 1995; Voskuil and Chambliss, 1998). Promoter-probe plasmid vectors, in which DNA fragments containing a putative promoter are fused to a promoter-less reporter gene (transcriptional fusions), are useful tools to demonstrate promoter activity *in vivo*. They are particularly necessary when dealing with bacterial genomes that have a high A+T content, as it is the case of *Streptococcus pneumoniae* (pneumococcus) and *Enterococcus faecalis* (enterococcus), whose genomes have about 60% of A+T content. In these genomes, stretches resembling –10 elements (5´-TATAAT-3´) are frequent and, therefore, definitive identification of promoters from sequence information alone remains more difficult.

The bacterial RNAP can terminate transcription efficiently at Rho-independent signals, which are active in the nascent transcript. These signals (also known as intrinsic terminators) typically consist of a G:C-rich stem-loop structure, followed by a short stretch of U residues. The stem-loop structure halts the RNAP and leads to its release. Thus, transcription termination occurs near the end of the poly(U) region. Furthermore, transcription attenuation is a highly conserved regulatory

mechanism used by bacteria. Attenuators are usually located at the 5´ untranslated regions of genes or operons and combine an intrinsic terminator with an RNA element that senses specific environmental stimuli (Merino and Yanofsky, 2005; Naville and Gautheret, 2009). Several algorithms are able to detect intrinsic terminators in genomic DNAs (de Hoon et al., 2005; d´Aubenton-Carafa et al., 1990; Kingsford et al., 2007; Lesnik et al., 2001). Nevertheless, some intrinsic terminating sequences deviate from the common motif and, consequently, the availability of terminator-probe plasmid vectors makes possible to rapidly test whether a particular sequence functions as a terminator signal *in vivo*.

The Gram-positive bacteria *S. pneumoniae* and *E. faecalis* are a leading cause of nosocomial infections. *S. pneumoniae* is normally found as a harmless commensal of the human upper respiratory tract. However, when the immune system weakens, it is also a major cause of life-threatening infections, such as pneumonia, meningitis and septicemia (Bogaert et al., 2004; Scott 2007). *E. faecalis* is a usual inhabitant of the gastrointestinal tract of humans and animals, but it can become an opportunistic pathogen and cause serious diseases, including bacteraemia, endocarditis and urinary tract infections (Amyes 2007; Murray and Weinstock, 1999). Pathogenic bacteria encounter diverse environments during the infectious cycle. Their ability to adapt efficiently to a new niche requires coordinated changes in the expression of multiple genes. In this context, promoter-probe and terminator-probe plasmid vectors are useful systems to investigate the expression of specific genes in a variety of genetic backgrounds and environmental stimuli. Despite this fact, such plasmid-based genetic tools are still very scarce in both S. *pneumoniae* and *E. faecalis*. In the present work, we describe the construction of novel promoter-probe and terminator-probe vectors

based on the *S. agalactiae* plasmid pMV158, which replicates autonomously in numerous Gram-positive bacteria (streptococci, enterococci, staphylococci, bacilli and lactococci). As reporter gene, we have used a variant of the *gfp* gene from the jellyfish *Aequorea victoria* (Miller and Lindow, 1997). We show that these vectors are suitable to assess whether particular sequences function as promoter or terminator signals in *S. pneumoniae* and *E. faecalis.* In addition, we show that the promoter-probe vector constitutes a valuable tool for the study of regulated promoters *in vivo* and, therefore, for the design of new inducible-expression vectors.

2. Materials and Methods

2.1. Bacterial strains and plasmids

S. pneumoniae 708 (*trt-1*, *hex-4*, *end-1*, *exo-2*, *malM594*) (Espinosa et al., 1982) and *E. faecalis* JH2-2 (resistant to rifampin and fusidic acid) (Jacob and Hobbs, 1974) were used as hosts for the plasmids constructed in this work. Genomic DNA was isolated from *S. pneumoniae* R61, a derivative of the R6 sequenced strain (Hoskins et al., 2001), and from *E. faecalis* V583, a clinical isolate resistant to vancomycin (Paulsen et al., 2003). In addition to the plasmids constructed in this work (see below), we used plasmid pLS1 (Lacks et al., 1986), a derivative of the streptococcal plasmid pMV158, the *E. coli* plasmid pGreenTIR (Miller and Lindow, 1997), a pUC1813 derivative that carries a *gfp* allele fused to an optimized translation initiation region, and the *Bacillus subtilis* plasmid pPR54 (Serrano-Heras et al., 2005), which carries the transcriptional termination sites of the *E. coli rrnB* ribosomal RNA operon (Brosius et al., 1981).

2.2. Growth and transformation of bacteria

The AGCH medium used for growth of *S. pneumoniae* was based on that described by Lacks (1966). It contains, per liter, 5 g acid-hydrolyzed casein (Difco), 1 g enzymatic casein hydrolysate (Pronadisa), 40 mg L-cysteine.HCl, 6 mg L-tryptophan, 50 mg L-asparagine, 10 mg L-glutamine, 5 mg adenine, 5 mg choline chloride, 1.2 mg calcium pantothenate, 0.3 mg nicotinic acid, 0.3 mg pyridoxine.HCl, 0.3 mg thiamine.HCl, 0.14 mg riboflavine, 0.6 μg biotin, 8.5 g K_2HPO_4, 2 g $NaC_2H_3O_2$, 0.4 g $NaHCO_3$, 0.5 g $MgCl_2.6H_2O$, 6 mg $CaCl_2$, 0.5 mg $FeSO_4.7H_2O$, 0.5 mg $CuSO_4.5H_2O$, 0.5 mg $ZnSO_4.7H_2O$, 0.2 mg $MnSO_4.4H_2O$, 0.5 g bovine albumin (Fraction V, Sigma), and 3000 units catalase (from *Aspergillus niger*, Calbiochem). For routine growth the AGCH medium was supplemented with 0.2% yeast extract (Difco) and 0.3% sucrose (Sigma). When indicated, other carbon sources were used. For the cultivation of *E. faecalis*, Bacto™ Brain Heart Infusion (BHI) medium was used. This medium was supplemented with 1.25% glycine when enterococcal cultures were grown for genomic DNA isolation. Pneumococcal and enterococcal cells containing pLS1-derivatives were grown in media supplemented with tetracycline at 1 and 4 μg/ml, respectively. All the experiments were performed at 37ºC. Procedures for competence development and transformation of *S. pneumoniae* were reported (Lacks et al., 1986). The protocol used to transform *E. faecalis* by electroporation was described (Shepard and Gilmore, 1995).

2.3. Total RNA preparations and primer extension

The Aurum Total RNA Mini Kit (BioRad) was used to isolate total RNA from *S. pneumoniae*. Plasmid-containing cells were grown as indicated above to an optical density at 650 nm (OD_{650}) of 0.2. Then, 3 ml of culture were processed as

specified by the supplier, except that the lysis solution was supplemented with 0.2% deoxycholate. The integrity of rRNAs was checked by agarose gel electrophoresis. The RNA concentration was determined using the NanoDrop ND-1000 Spectrophotometer. For primer extension, the ThermoScript Reverse Transcriptase enzyme (Invitrogen) and [α-^{32}P]-dATP (3000 Ci/mmol; Hartmann) were used. The reaction mixture was incubated at 50ºC for 45 min. Non-incorporated nucleotide was removed using MicroSpin G-25 columns (GE Healthcare). Samples were dried in a Speed Vac, resuspended in loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylenecyanol), and subjected to electrophoresis in a 8 M urea / 6% polyacrylamide gel. Dideoxy-mediated chain-termination sequencing reactions using DNA from M13mp18 (Yanisch-Perron et al., 1985) and the –40 M13 primer (5´-GTTTTCCCAGTCACGAC-3´) were run in the same gel.

2.4. Isolation of DNA

For small-scale preparations of purified plasmid DNA, the High Pure Plasmid Isolation Kit (Roche Applied Science) was used. The *Suspension Buffer* of this kit was supplemented with 50 mM glucose and 0.1% deoxycholate in pneumococcus, or with 50 mM glucose, 700 μg/ml lysozyme and 240 units/ml mutanolysin in enterococcus. Genomic DNA from *S. pneumoniae* was prepared as previously described (Lacks, 1966). To isolate genomic DNA from *E. faecalis*, cultures at an OD_{650} of 1.2 were concentrated 10-fold in buffer A (25% sucrose, 0.1 M NaCl, 50 mM Tris-HCl, pH 8.0, 28 μg/ml RNase A, 10 mg/ml lysozyme). Then, mutanolysin (150 units) was added to 1 ml of the concentrated culture. After 20 min at 37ºC, SDS was added at a final concentration of 1%. The lysate was treated with proteinase K (240 μg/ml) for 15 min. DNA was further purified by extraction with

phenol/chloroform, dialyzed against buffer TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and recovered by precipitation with ethanol.

2.5. Polymerase chain reaction (PCR) conditions

Phusion High-Fidelity DNA Polymerase (Finnzymes) was used for all PCR applications. The Phusion HF Buffer provided by the manufacturer was used as reaction buffer. The reaction mixtures (50 µl) contained 5-30 ng of template DNA, 20-30 pmoles of each primer, 200 µM of each dNTP and 1 unit of DNA polymerase. An initial denaturation step was performed at 98ºC for 1 min. Then, it was followed by 30 cycles that included the next steps: (i) denaturation at 98ºC for 10 s; (ii) annealing at around 55ºC (depending on the primer Tm) for 20 s and (iii) extension at 72ºC for 40 s. A final extension step was performed at 72ºC for 10 min.

2.6. Construction of plasmids pAS and pSA

To construct the terminator-probe vector pAS, an 833-bp region of the pGreenTIR plasmid (Miller and Lindow, 1997), which contains the *gfp* reporter cassette, was amplified by PCR with the F-*gfp* and R-*gfp* oligonucleotides (Table 1). Both of them include a *Hind*III restriction site. Then, the PCR-amplified DNA was purified and digested with *Hind*III, generating an 802-bp DNA fragment. The QIAquick PCR Purification Kit (QIAGEN) was used to purify DNA from both PCR and restriction endonuclease digestion. The 802-bp *Hind*III fragment was mixed with *Hind*III-linearized pLS1 DNA (Lacks et al., 1986). The mixture was treated with T4 DNA ligase (New England Biolabs) and used to transform competent *S. pneumoniae* 708 cells. Transformants were selected for tetracycline (1 µg/ml) at 37ºC. Subsequently, plasmid DNA was isolated and analyzed by restriction

mapping. In the recombinant plasmid pAS, the *tetL* (resistance to tetracycline) and *gfp* genes are located on the same DNA strand. Plasmid pSA carries the inserted fragment in the opposite orientation. To confirm the constructions, the inserted fragment and the regions of pLS1 that are flanking the insert were sequenced. Dye-terminator sequencing was carried out at Secugen (Centro de Investigaciones Biológicas, Madrid).

2.7. PCR-amplification of transcriptional terminator regions

Primers used for PCR-amplification of terminator regions are listed in Table 1. For PCR-amplification of a 286-bp region that contains the transcriptional termination sites *T1T2* of the *E. coli rrnB* ribosomal RNA operon (Brosius et al., 1981), we used the pPR54 plasmid (Serrano-Heras et al., 2005) as template and the F-*T1T2rrnB* and R-*T1T2rrnB* oligonucleotides as primers. The PCR-synthesized DNA was further digested with *Sal*I, and the 246-bp digestion product was inserted into the *Sal*I site of plasmid pAS in both orientations: plasmid pAST (orientation *T1T2rrnB*; promoter-probe vector) and plasmid pAS-*T2T1rrnB* (opposite orientation). For the construction of plasmid pAS-*TpolA*, a 278-bp region of the pneumococcal genome containing the terminator of the *polA* gene (López et al., 1989) was amplified with the F-*TpolA* and R-*TpolA* primers. After *Sal*I digestion, the generated 238-bp fragment was cloned into the *Sal*I site of the pAS vector. For the construction of plasmid pAS-*TrsiV*, a 305-bp region of the enterococcal genomic DNA that contains the putative terminator of the *sigV-rsiV* operon (Benachour et al., 2005) was amplified with the F-*TrsiV* and R-*TrsiV* primers. Then, the PCR-amplified DNA was digested with *Sal*I, and the 265-bp generated fragment was inserted into the *Sal*I site of the pAS vector.

2.8. PCR-amplification of promoter regions

Primers used for PCR-amplification of promoter regions are listed in Table 1. Using pneumococcal genomic DNA as template, two regions of 199-bp and 195-bp containing the promoter of the *sulA* (Lacks et al., 1995; López et al., 1987) and *ung* (Méjean et al., 1990) genes, respectively, were amplified with the F-*PsulA* and R-*PsulA* primers or the F-*Pung* and R-*Pung* primers. The PCR-synthesized DNAs were further digested with *Bam*HI. The 166-bp (*PsulA* promoter) and 159-bp (*Pung* promoter) digestion products were inserted into the *Bam*HI site of the pAST vector, generating plasmids pAST-*PsulA* and pAST-*Pung*, respectively. From the enterococcal genome, two regions of 192-bp and 190-bp containing the promoter of the *uppS* and *EF2493* genes (Hancock et al., 2003), respectively, were amplified with the F-*PuppS* and R-*PuppS* primers or the F-*P2493* and R-*P2493* primers. After *Sac*I digestion, the 164-bp (*PuppS* promoter) and 160-bp (*P2493* promoter) restriction fragments were cloned into the *Sac*I site of the pAST vector, generating plasmids pAST-*PuppS* and pAST-*P2493*, respectively. Moreover, to construct plasmid pAST-*P2962*, a 191-bp region of the enterococcal genome that contains the putative promoter of the *EF2962* gene was amplified with the F-*P2962* and R-*P2962* primers. After *Bam*HI digestion, the 158-bp restriction fragment (*P2962* promoter) was inserted into the *Bam*HI site of the pAST vector. Concerning the pneumococcal *PfcsK* promoter, a 150-bp region was amplified using genomic DNA as template and the oligonucleotides F-*PfcsK* and R-*PfcsK* as primers. After *Xba*I digestion, the 117-bp restriction fragment (*PfcsK* promoter) was cloned into the *Xba*I site of the pAST vector in both orientations: plasmid pAST-*PfcsK* (gene *gfp* under the control of the *PfcsK* promoter) and plasmid pAST-o*PfcsK* (opposite orientation).

2.9. Fluorescence assays

Pneumococcal and enterococcal cells carrying plasmid were grown as indicated to an OD_{650} of 0.3 (logarithmic phase), except in the study of the pneumococcal *PfcsK* promoter. In this case, bacteria were grown to an OD_{650} of 0.6 (late logarithmic phase), since fucose-induced expression from the *PfcsK* promoter was reported to increase strongly during such a phase (Chan et al., 2003). Then, different volumes of the culture (25 µl to 1 ml) were centrifuged, and cells were resuspended in 200 µl of PBS buffer (10 mM Na_2HPO_4, 1 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl, pH 7.2). Fluorescence was measured on a LS-50B Luminescence Spectrometer (Perkin-Elmer) by excitation at 488 nm with a slit width of 15 nm and detection of emission at 515 nm with a slit width of 7.5 nm. In each case, three independent cultures were analyzed. The fluorescence corresponding to 200 µl of PBS buffer without cells was around 40 arbitrary units.

2.10. Western blots

Plasmid-carrying pneumococcal cells were grown as indicated to late logarithmic phase (OD_{650} = 0.6). Media contained 0.3% sucrose and different concentrations of fucose (0.1% to 1%) as carbon source. To prepare whole-cell extracts, bacteria were concentrated 40-fold in buffer L (50 mM Tris-HCl, pH 7.6, 1 mM EDTA, 50 mM NaCl, 0.1% deoxycholate), and incubated at 30ºC for 10 min. Then, a sample (8 µl) of each cell extract was mixed with 2 µl of 5x loading buffer (250 mM Tris-HCl, pH 6.8, 10% SDS, 25% β-mercaptoethanol, 50% glycerol, 0.5% bromophenol blue), and total proteins were separated by SDS-polyacrylamide gel electrophoresis (14% polyacrylamide). Thus, equivalent amounts of the cell extracts (similar number of cells) were loaded onto the gel. Pre-stained proteins

(Invitrogen) were run in the same gel as molecular weight markers. Proteins were transferred electrophoretically to Immun-blot PVDF membranes (BioRad) using a Mini Trans Blot (Bio-Rad) at 100 mA and 4ºC for 90 min. Transfer buffer contained 25 mM Tris, 192 mM glycine, 20% methanol. Anti-GFP (Roche Applied Science), a mixture of two mouse monoclonal antibodies against the green fluorescent protein, was used as specified by the supplier. Antigen-antibody complexes were detected using peroxidase-conjugated AffiniPure Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch), the Immun-StarTM HRP Substrate Kit (BioRad), and the Luminescent Image Analyzer LAS-3000 (Fujifilm Life Science). The intensity of the bands was quantified using the QuantityOne software (BioRad).

3. Results and Discussion

3.1. Transcription through the HindIII site in plasmid pLS1

The streptococcal plasmid pMV158 (5540 bp), which is the prototype of a family of rolling-circle replicating plasmids, is able to replicate in a broad variety of bacterial hosts (del Solar et al., 1998). Moreover, it confers resistance to tetracycline (*tetL* gene) in both Gram-positive and Gram-negative bacteria. Sequence analysis of the region located just downstream of the *tetL* gene revealed the existence of an inverted-repeat (IR in Fig. 1) followed by a short stretch of thymine residues (Lacks et al., 1986). This sequence element has the features of a Rho-independent transcriptional terminator and is also present in plasmid pLS1 (Fig.1), a pMV158-derivative that lacks the 1132-bp *Eco*RI restriction fragment (Lacks et al., 1986). To analyze the efficiency of the *tetL* inverted-repeat as transcriptional terminator, we investigated whether continuation of transcription occurs at the *tetL* inverted-repeat in *S. pneumoniae* cells. If this were the case, mRNA molecules containing the sequence termed INT in Fig. 1 should be synthesized. Such molecules could form a stem-loop structure followed by a poly(U) region. To this end, the INTc oligonucleotide (Table 1), whose sequence is complementary to the INT region, was used as primer for extension on total RNA isolated from pLS1-carrying *S. pneumoniae* cells. As shown in Fig. 1, two cDNA extension products of 106 and 107 nucleotides were detected. These products are likely generated by reverse transcriptase pausing at the base of the potential RNA stem-loop structure rather than by reverse transcriptase running off at 5´ ends of newly initiated transcripts. In fact, promoter sequences just upstream of the poly(T) region are not predicted. Transcription through the INT region was further confirmed by cloning a *gfp* reporter cassette into the *Hind*III site of plasmid

pLS1 (Fig. 2). The cassette was inserted in both orientations (plasmids pAS and pSA). In plasmid pAS, the *tetL* and *gfp* genes are located on the same DNA strand. The *gfp* reporter cassette contains a multiple cloning site (MCS) followed by a promoter-less *gfp* allele, which encodes a green fluorescent protein (GFP) that carries the F64L and S65T mutations (Cormack et al., 1996; Heim et al., 1995). The F64L mutation increases GFP solubility, while the S65T mutation increases GFP fluorescence and causes a red shift in the excitation spectrum. In addition, the *gfp* allele carries translation initiation signals (SD in Fig. 2) that are optimal for its expression in prokaryotes (Miller and Lindow, 1997). Plasmid pSA carries the *gfp* reporter cassette in the opposite orientation. First, we analyzed *gfp* expression in *S. pneumoniae* 708 cells carrying the pAS or pSA plasmid by measuring the intensity of fluorescence at 515 nm (excitation at 488 nm) (Fig. 2). No *gfp* gene expression was observed in pSA-harbouring cells, which confirms the absence of promoter signals within the *gfp* reporter cassette. However, the fluorescence increased as a function of the culture volume in cells harbouring plasmid pAS. The fluorescence corresponding to 0.8 ml culture (OD_{650} = 0.3) was 3-fold higher than the background level (pSA-containing cells). Plasmids pAS and pSA were further introduced into *E. faecalis* JH2-2 cells. As expected, *gfp* expression was detected only in pAS-carrying cells. Specifically, the fluorescence of 0.8 ml culture (OD_{650} = 0.3) was 4.5-fold higher than the background level. From these results, we conclude that both the pneumococcal and enterococcal RNA polymerases are able to transcribe through the *tetL* inverted-repeat of the pLS1 plasmid (Fig. 1) and, therefore, to transcribe the *gfp* reporter cassette inserted into its *Hind*III site (Fig. 2). This fact and the presence of a MCS between the *Hind*III

site and the promoter-less *gfp* gene make plasmid pAS a useful terminator-probe vector (see below).

3.2. Use of plasmid pAS as a terminator-probe vector in S. pneumoniae and E. faecalis

To analyze whether plasmid pAS (5210 bp) is useful for the detection of transcriptional terminator signals, we selected some predicted or experimentally determined Rho-independent terminators from different bacterial genomes. Specifically, we inserted independently the following DNA sequences (Fig. 3) into the *Sal*I site of the pAS plasmid (see Fig. 2): (i) a 246-bp *Sal*I restriction fragment containing the tandem terminators *T1* and *T2* of the *E. coli rrnB* ribosomal RNA operon (Brosius et al., 1981). Such a fragment was inserted in both orientations (herein termed *T1T2rrnB* and *T2T1rrnB* fragments, respectively). These terminators have been used frequently in the construction of plasmid vectors (Brosius, 1984; Serrano-Heras et al., 2005; Simons et al., 1987); (ii) a 238-bp *Sal*I restriction fragment containing the transcriptional terminator of the *S. pneumoniae polA* gene (referred to as *TpolA* fragment). By mapping with S1 nuclease, it was shown that transcription of the *polA* gene terminates at the palindrome shown in Fig. 3 (López et al., 1989); and (iii) a 265-bp *Sal*I restriction fragment containing the putative Rho-independent terminator of the *E. faecalis sigV-rsiV* operon (herein termed *TrsiV* fragment). The *sigV* and *rsiV* genes encode members of the extracytoplasmic function subfamily of eubacterial RNA polymerase sigma and anti-sigma factors, respectively (Benachour et al., 2005). All the recombinant plasmids (named pAST, pAS-*T2T1rrnB*, pAS-*TpolA* and pAS-*TrsiV*) were introduced into *S. pneumoniae* 708 and *E. faecalis* JH2-2 cells. The efficiency of the inserted fragments as transcriptional terminators was evaluated by monitoring

gfp gene expression (Table 2). The fluorescence in pneumococcal and enterococcal cells carrying the control plasmid pSA (background level) was 46.32 ± 2.24 and 58.08 ± 0.64, respectively. Compared to pAS-carrying cells, the *T1T2rrnB* and *TrsiV* fragments reduced the intensity of fluorescence to background values in both *S. pneumoniae* and *E. faecalis*. In the case of the *T2T1rrnB* fragment, the fluorescence decreased 1.8 and 1.5-fold in pneumococcus and enterococcus, respectively. However, the *TpolA* fragment reduced the fluorescence in *E. faecalis* (3-fold) but not in *S. pneumoniae*. A further analysis of the *TpolA* fragment using the BPROM prediction program (*Softberry, Inc.*) revealed a near-consensus –10 hexamer (TAgAAT) located 5 nucleotides downstream of the *TpolA* palindrome, as well as a near-consensus extended –10 element (TGTa) (see Fig. 3). Thus, activity of this predicted promoter in *S. pneumoniae* but not in *E. faecalis* might explain why the terminator activity of the *TpolA* palindrome was only detected in *E. faecalis*. In conclusion, these results demonstrate that plasmid pAS can be used to examine whether particular sequences (homologous or heterologous) function as transcriptional terminators in *S. pneumoniae* and *E. faecalis*. Moreover, we have shown that the predicted *TrsiV* terminator of *E. faecalis* is active in both bacteria. In our system, it is as efficient as the tandem terminators *T1* and *T2* of *E. coli*.

3.3. Use of plasmid pAST as a promoter-probe vector in S. pneumoniae and E. faecalis

Promoters recognized by RNAP holoenzymes that carry a σ-factor similar to *E. coli* σ^{70} are characterized by two elements, the –35 (consensus 5´-TTGACA-3´) and –10 (consensus 5´-TATAAT-3´) hexamers (Haugen et al., 2008). In addition,

some of these promoters contain an extended –10 element (5´-TRTG-3´ motif in Gram-positive bacteria) (Sabelnikov et al., 1995; Voskuil and Chambliss, 1998). Since the sequence elements at numerous promoters have evolved to diverge from the consensus, definitive identification of a promoter target for RNAP requires the use of diverse experimental strategies, such as the use of promoter-probe plasmid vectors (reviewed in Minchin and Busby, 2009; Ross and Gourse, 2009).

Cloning of the *E. coli T1T2rrnB* terminator region into the *Sal*I site of the pAS terminator-probe vector generated plasmid pAST (5456 bp; see above). This derivative conserves unique restriction sites (*Xba*I, *Bam*HI, *Sma*I, *Sac*I) between the *T1T2rrnB* region and the promoter-less *gfp* gene (see Fig. 2). To investigate whether plasmid pAST is suitable as a promoter-probe vector, we selected several DNA fragments containing a predicted or experimentally tested promoter from *S. pneumoniae* or *E. faecalis* (Fig. 4). These promoter regions were independently inserted into the *Bam*HI or *Sac*I site of the pAST plasmid (for details see Materials and Methods). The recombinant plasmids were then introduced into *S. pneumoniae* 708 and *E. faecalis* JH2-2 cells, and promoter activity was evaluated by monitoring *gfp* expression (fluorescence assays) (Table 3). Regarding pneumococcal promoters, we analyzed the promoter region of the *sulA* (dihydropteroate synthase) and *ung* (uracil-DNA glycosylase) genes. The *PsulA* promoter, which was identified by primer extension (Lacks et al., 1995; López et al., 1987), has a near-consensus –10 hexamer and a consensus –10 extension (Fig. 4). In the case of the *ung* gene (Méjean et al., 1990), the BPROM prediction program (*Softberry, Inc.*) revealed a consensus –10 hexamer, which is located 28 nucleotides upstream of the translation initiation codon, and a near-consensus –10 extension (Fig. 4). In pneumococcus, and compared to pAST-containing cells

(46.08 ± 3.99 units), the intensity of fluorescence increased 10-fold when the *Pung* promoter region was inserted into pAST (plasmid pAST-*Pung*) (Table 3). The activity of such a promoter was 1.9-fold higher than that of the *PsulA* promoter. Different results were obtained in enterococcus. In this case, and compared to cells carrying pAST (58.92 ± 2.64 units), the fluorescence increased only 2-fold in cells harbouring the pAST-*Pung* recombinant plasmid. Moreover, the activity of the *Pung* promoter was slightly lower than that of the *PsulA* promoter. We further analyzed the promoter region of three genes from *E. faecalis* V583 (Paulsen et al., 2003): *uppS* (or *cpsA*; undecaprenyl diphosphate synthase) (Hancock and Gilmore, 2002; Thurlow et al., 2009), *EF2493* (or *cpsC*; putative teichoic acid biosynthesis protein) (Hancock and Gilmore, 2002) and *EF2962* (putative LacI family transcriptional regulator). The *PuppS* and *P2493* promoters were identified by primer extension (Hancock et al., 2003). The *PuppS* promoter has a consensus –10 hexamer and shows a 4/6 match at the –35 element, whereas the *P2493* promoter has near-consensus –10 and –35 hexamers (Fig. 4). In the case of the *EF2962* gene, the BPROM program predicted a –10 hexamer (four consensus bases) located 56 nucleotides upstream of the initiation codon. This promoter has a near-consensus –10 extension and shows a 3/6 match at the –35 element (Fig. 4). As shown in Table 3, the *P2493* promoter was the strongest enterococcal promoter in both *S. pneumoniae* and *E. faecalis*. In pneumococcus, the activity of the *P2493* promoter was 1.6 and 2.3-fold higher than that of the *PuppS* and *P2962* promoters, respectively. In enterococcus, and compared to the *P2493* promoter, the activity of the *PuppS* and *P2962* promoters was 5.2 and 3.2-fold lower, respectively. Therefore, plasmid pAST can be used to assess the activity of specific promoter sequences (homologous and heterologous) in *S. pneumoniae*

and *E. faecalis*. Among the analyzed promoters, we have shown that two predicted promoters, *Pung* and *P2962*, are active in both bacteria. Furthermore, we have demonstrated that, under our experimental conditions, the strongest promoters (10-fold increase in fluorescence) are the *Pung* promoter in pneumococcus and the *P2493* promoter in enterococcus. We conclude that plasmid pAST is a useful vector for *in vivo* studies of promoter sequences.

3.4. Fucose-regulation of the pneumococcal PfcsK promoter cloned into the pAST vector

The promoter of the pneumococcal fuculose kinase gene (*fcsK*), the first gene of the fucose operon, is induced by fucose (Chan et al., 2003). This promoter (*PfcsK*) has a canonical –10 hexamer and a near-consensus –35 sequence (TTGAaA). Both sequence elements are separated by 17 nucleotides. According to primer extension experiments, transcription of the *fcsK* gene starts at an adenine residue located 24 nucleotides upstream of the initiation codon (Chan et al., 2003). To determine whether plasmid pAST constitutes a valuable tool for the study of regulated promoters, a 117-bp *Xba*I restriction fragment containing the *PfcsK* promoter was inserted into the *Xba*I site of the pAST vector, generating plasmids pAST-*PfcsK* (gene *gfp* under the control of the *PfcsK* promoter) and pAST-o*PfcsK* (opposite orientation). Both recombinant plasmids were introduced into the *S. pneumoniae* 708 strain, which is thought to have a single chromosomal copy of the putative fucose regulator gene *fcsR*. Then, we examined whether fucose induces *gfp* expression in cells carrying the pAST-*PfcsK* plasmid. Cells harbouring pAST-o*PfcsK* were used as control. Since *S. pneumoniae* is unable to grow in media containing fucose as the sole carbon source (Chan et al., 2003), bacteria were grown in media containing 0.3% sucrose and different

concentrations of fucose to late logarithmic phase (OD_{650} = 0.6). The bacterial growth rate was similar under the various conditions assayed (not shown). In a first approach, *gfp* expression was analyzed by Western blotting using monoclonal GFP antibodies (Fig. 5A). A protein band was detected in cells carrying pAST-*PfcsK* but not in control cells (plasmid pAST-o*PfcsK*). Since pre-stained proteins were run in the same gel, exposition of the blot to X-ray films allowed us to determine that such a band had the mobility expected for GFP (~ 28 kDa) (not shown). The Western blot analysis revealed a basal level of *gfp* expression in cells grown without fucose, suggesting that a single chromosomal copy of the putative fucose regulator gene *fcsR* is not sufficient for total repression of the *PfcsK* promoter placed on a pLS1 derivative (pLS1 has ~22 copies per genome equivalent, del Solar et al., 1993). However, compared to cells grown without fucose, the intensity of the GFP band was 4.5-fold higher in cells grown with 1% fucose. Hence, the *PfcsK* promoter cloned into the pAST vector is activated by fucose. These results were further confirmed by fluorescence assays (Fig. 5B). In the absence of fucose, the fluorescence in cells carrying pAST-*PfcsK* (64.66 ± 5.95 units) was slightly higher than in cells harbouring pAST-o*PfcsK* (43.22 ± 2.30; control cells). Thus, there is a low basal level of *gfp* expression. Moreover, the fluorescence in cells carrying pAST-*PfcsK* increased as a function of the fucose concentration (from 0.1% to 1%). Specifically, a 5-fold increase in fluorescence was observed when the medium was supplemented with 1% fucose (Fig. 5B). Under those conditions, no changes were detected in the fluorescence of the control cells (41.15 ± 1.81 units with 1% fucose). Since the fucose operon and the putative fucose regulator gene *fcsR* are widely conserved in *S. pneumoniae* (Weng et al., 2009), it is to be expected that plasmid pAST-*PfcsK* will be valuable

as inducible-expression vector in pneumococcus. Our results concerning the *PfcsK* promoter support that the promoter-probe vector pAST can be used to detect growth conditions that favour the expression of a particular regulated promoter.

To conclude, the promoter-probe and terminator-probe vectors described in this work are suitable to assess the activity of promoter and terminator signals (both homologous and heterologous) in *S. pneumoniae* and *E. faecalis*. These vectors are based on pMV158, which is one of the most promiscuous replicons reported so far. It has been established in nearly 30 different bacterial species (M. E., unpublished observations). Hence, it is very likely that these newly constructed plasmid-based genetic tools can be used in a number of Gram-positive bacteria. Furthermore, employment of some of the promoters tested here could be useful when constructing strains that would express a desired genetic trait.

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References

Amyes, S.G.B., 2007. Enterococci and streptococci. Int. J. Antimicrob. Agents 29, S43-S52.

Askary, A., Masoudi-Nejad, A., Sharafi, R., Mizbani, A., Parizi, S.N., Purmasjedi, M., 2009. N4: A precise and highly sensitive promoter predictor using neural network fed by nearest neighbors. Genes Genet. Syst. 84, 425-430.

Benachour, A., Muller, C., Dabrowski-Coton, M., Le Breton, Y., Giard, J., Rincé, A., Auffray, Y., Hartke, A., 2005. The *Enterococcus faecalis* SigV protein is an extracytoplasmic function sigma factor contributing to survival following heat, acid, and ethanol treatments. J. Bacteriol. 187, 1022-1035.

Bogaert, D., de Groot R., Hermans, P.W.M., 2004. *Streptococcus pneumoniae* colonisation: the key to pneumococcal disease. Lancet Infect. Dis. 4, 144-154.

Brosius, J., 1984. Plasmid vectors for the selection of promoters. Gene 27, 151-160

Brosius, J., Dull, T.J., Sleeter, D.D., Noller, H.F., 1981. Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. J. Mol. Biol. 148, 107-127.

Chan, P.F., O'Dwyer, K.M., Palmer, L.M., Ambrad, J.D., Ingraham, K.A., So, C., Lonetto, M.A., Biswas, S., Rosenberg, M., Holmes, D.J., Zalacain, M., 2003. Characterization of a novel fucose-regulated promoter (*PfcsK*) suitable for gene essentiality and antibacterial mode-of-action studies in *Streptococcus pneumoniae*. J. Bacteriol. 185, 2051-2058.

Chuang, L.Y., Tsai, J.H., Yang, C.H., 2010. Binary particle swarm optimization for operon prediction. Nucl. Acids Res. 38, e128.

Cormack, B.P., Valdivia, R.H., Falkow, S., 1996. FACS-optimized mutants of the green fluorescent protein (GFP). Gene 173, 33-38.

de Hoon, M.J.L., Makita, Y., Nakai, K., Miyano, S., 2005. Prediction of transcriptional terminators in *Bacillus subtilis* and related species. PLoS Comput. Biol. 1, e25.

del Solar, G., Kramer, G., Ballester, S., Espinosa, M., 1993. Replication of the promiscuous plasmid pLS1: a region encompassing the minus origin of replication is associated with stable plasmid inheritance. Mol. Gen. Genet. 241, 97-105.

del Solar, G., Giraldo, R., Ruiz-Echevarria, M.J., Espinosa, M., Díaz-Orejas, R., 1998. Replication and control of circular bacterial plasmids. Microbiol. Mol. Biol. Rev. 62, 434-464.

d´Aubenton-Carafa, Y., Brody, E., Thermes, C., 1990. Prediction of Rho-independent *Escherichia coli* transcription terminators. A statistical analysis of their RNA stem-loop structures. J. Mol. Biol. 216, 835-858.

Espinosa, M., López, P., Pérez-Ureña, M.T., Lacks, S.A., 1982. Interspecific plasmid transfer between *Streptococcus pneumoniae* and *Bacillus subtilis*. Mol. Gen. Genet. 188, 195-201.

Gruber, T.M., Gross, C.A., 2003. Multiple sigma subunits and the partitioning of bacterial transcription space. Annu. Rev. Microbiol. 57, 441-466.

Hancock, L.E., Gilmore, M.S., 2002. The capsular polysaccharide of *Enterococcus faecalis* and its relationship to other polysaccharides in the cell wall. Proc. Natl. Acad. Sci. USA 99, 1574-1579.

Hancock, L.E., Shepard, B.D., Gilmore, M.S., 2003. Molecular analysis of the *Enterococcus faecalis* serotype 2 polysaccharide determinant. J. Bacteriol. 185, 4393-4401.

Haugen, S.P., Ross, W., Gourse, R.L., 2008. Advances in bacterial promoter recognition and its control by factors that do not bind DNA. Nat. Rev. Microbiol. 6,

507-516.

Heim, R., Cubitt, A.B., Tsien, R.Y., 1995. Improved green fluorescence. Nature 373, 663-664.

Hoskins, J., Alborn, W.E. Jr., Arnold, J., Blaszczak, L.C., Burgett, S., DeHoff, B.S., Estrem, S.T., Fritz, L., Fu, D.-J., Fuller, W., Geringer, C., Gilmour, R., Glass, J.S., Khoja, H., Kraft, A.R., Lagace, R.E., LeBlanc, D.J., Lee, L.N., Lefkowitz, E.J., Lu, J., Matsushima, P., McAhren, S.M., McHenney, M., McLeaster, K., Mundy, C.W., Nicas, T.I., Norris, F. H., O'Gara, M., Peery, R.B., Robertson, G.T., Rockey, P., Sun, P.-M., Winkler, M.E., Yang, Y., Young-Bellido, M., Zhao, G., Zook, C.A., Baltz, R.H., Jaskunas, S.R., Rosteck, P.R. Jr., Skatrud, P.L., Glass, J.I., 2001. Genome of the bacterium *Streptococcus pneumoniae* strain R6. J. Bacteriol. 183, 5709-5717.

Jacob, A.E., Hobbs, S.J., 1974. Conjugal transfer of plasmid-borne multiple antibiotic resistance in *Streptococcus faecalis* var. *zymogenes*. J. Bacteriol. 117, 360-372.

Jacques, P.E., Rodrigue, S., Gaudreau, L., Goulet, J., Brzezinski, R., 2006. Detection of prokaryotic promoters from the genomic distribution of hexanucleotide pairs. BMC Bioinformatics 7, 423-436.

Kingsford, C.L., Ayanbule, K., Salzberg, S.L., 2007. Rapid, accurate, computational discovery of Rho-independent transcription terminators illuminates their relationship to DNA uptake. Genome Biol. 8, R22.

Lacks, S.A., 1966. Integration efficiency and genetic recombination in pneumococcal transformation. Genetics 53, 207-235.

Lacks, S.A., Greenberg, B., López, P., 1995. A cluster of four genes encoding enzymes for five steps in the folate biosynthetic pathway of *Streptococcus pneumoniae*. J. Bacteriol. 177, 66-74.

Lacks, S.A., López, P., Greenberg, B., Espinosa, M., 1986. Identification and analysis of genes for tetracycline resistance and replication functions in the broad-host-range plasmid pLS1. J. Mol. Biol. 192, 753-765.

Lesnik, E.A., Sampath, R., Levene, H.B., Henderson, T.J., McNeil, J.A., Ecker, D.J., 2001. Prediction of Rho-independent transcriptional terminators in *Escherichia coli*. Nucl. Acids Res. 29, 3583-3594.

López, P., Espinosa, M., Greenberg, B., Lacks, S.A., 1987. Sulfonamide resistance in *Streptococcus pneumoniae*: DNA sequence of the gene encoding dihydropteroate synthase and characterization of the enzyme. J. Bacteriol. 169, 4320-4326.

López, P., Martínez, S., Díaz, A., Espinosa, M., Lacks, S.A., 1989. Characterization of the *polA* gene of *Streptococcus pneumoniae* and comparison of the DNA polymerase I it encodes to homologous enzymes from *Escherichia coli* and phage T7. J. Biol. Chem. 264, 4255-4263.

Méjean, V., Rives, I., Claverys, J.P., 1990. Nucleotide sequence of the *Streptococcus pneumoniae ung* gene encoding uracil-DNA glycosylase. Nucl. Acids Res. 18, 6693.

Merino, E., Yanofsky C., 2005. Transcription attenuation: a highly conserved regulatory strategy used by bacteria. Trends Genet. 21, 260-264.

Miller, W.G., Lindow, S.E., 1997. An improved GFP cloning cassette designed for prokaryotic transcriptional fusions. Gene 191, 149-153.

Minchin, S.D., Busby, S.J.W., 2009. Analysis of mechanisms of activation and repression at bacterial promoters. Methods 47, 6-12.

Mitchell, J.E., Zheng, D., Busby, S.J.W., Minchin, S.D., 2003. Identification and analysis of 'extended –10' promoters in *Escherichia coli*. Nucl. Acids Res. 31,

4689-4695.

Murray, B.E., Weinstock, G.M., 1999. Enterococci: new aspects of an old organism. Proc. Assoc. Am. Physicians 111, 328-334.

Naville, M., Gautheret, D., 2009. Transcription attenuation in bacteria: theme and variations. Brief. Funct. Genomic Proteomic 8, 482-492.

Paulsen, I.T., Banerjei, L., Myers, G.S.A., Nelson, K.E., Seshadri, R., Read, T.D., Fouts, D.E., Eisen, J.A., Gill, S.R., Heidelberg, J.F., Tettelin, H., Dodson, R.J., Umayam, L., Brinkac, L., Beanan, M., Daugherty, S., DeBoy, R.T., Durkin, S., Kolonay, J., Madupu, R., Nelson, W., Vamathevan, J., Tran, B., Upton, J., Hansen, T., Shetty, J., Khouri, H., Utterback, T., Radune, D., Ketchum K.A., Dougherty, B.A., Fraser, C.M., 2003. Role of mobile DNA in the evolution of vancomycin-resistant *Enterococcus faecalis*. Science 299, 2071-2074.

Ross, W., Gourse, R.L., 2009. Analysis of RNA polymerase-promoter complex formation. Methods 47, 13-24.

Sabelnikov, A.G., Greenberg, B., Lacks, S.A., 1995. An extended -10 promoter alone directs transcription of the *DpnII* operon of *Streptococcus pneumoniae*. J. Mol. Biol. 250, 144-155.

Scott, J.A.G., 2007. The preventable burden of pneumococcal disease in the developing world. Vaccine 25, 2398-2405.

Serrano-Heras, G., Salas, M., Bravo, A., 2005. A new plasmid vector for regulated gene expression in *Bacillus subtilis*. Plasmid 54, 278-282.

Shepard, B.D., Gilmore, M.S., 1995. Electroporation and efficient transformation of *Enterococcus faecalis* grown in high concentrations of glycine. Methods Mol. Biol. 47, 217-226.

Simons, R.W., Houman, F., Kleckner, N., 1987. Improved single and multicopy *lac*-

based cloning vectors for protein and operon fusions. Gene 53, 85-96.

Taboada, B., Verde, C., Merino, E., 2010. High accuracy operon prediction method based on STRING database scores. Nucl. Acids Res. 38, e130.

Thurlow, L.R., Thomas, V.C., Hancock, L.E., 2009. Capsular polysaccharide production in *Enterococcus faecalis* and contribution of CpsF to capsule serospecificity. J. Bacteriol. 191, 6203-6210.

Voskuil, M.I., Chambliss, G.H., 1998. The -16 region of *Bacillus subtilis* and other gram-positive bacterial promoters. Nucl. Acids Res. 26, 3584-3590.

Weng, L., Biswas, I., Morrison, D.A., 2009. A self-deleting Cre-lox-ermAM cassette, Cheshire, for marker-less gene deletion in *Streptococcus pneumoniae*. J. Microbiol. Methods 79, 353-357.

Wigneshweraraj, S., Bose, D., Burrows, P.C., Joly, N., Schumacher, J., Rappas, M., Pape, T., Zhang, X., Stockley, P., Severinov, K., Buck, M., 2008. *Modus operandi* of the bacterial RNA polymerase containing the σ^{54} promoter-specificity factor. Mol. Microbiol. 68, 538-546.

Yanisch-Perron, C., Vieira, J., Messing, J., 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33, 103-119.

Figure legends

Fig. 1. Primer extension on total RNA isolated from pLS1-carrying pneumococcal cells. *copG* and *repB* are genes involved in plasmid DNA replication. The location of the replication origins *dso* (double-strand origin) and *ssoA* (single-strand origin) is indicated. The nucleotide sequence of the region spanning the translation stop codon (TAA) of the *tetL* gene and the *Hind*III site (H) is shown. IR: inverted-repeat, E: *Eco*RI site. The INTc oligonucleotide (see Table 1), whose sequence is complementary to the INT region, was used as primer. The asterisks indicate the 3´-end of the cDNA products (P) generated by the reverse transcriptase. A, C, G, T sequence ladders were used as DNA size markers. Specifically, dideoxy-mediated chain-termination sequencing reactions using DNA from M13mp18 and the –40 M13 primer (5´-GTTTTCCCAGTCACGAC-3´) were run in the same gel. A partial sequence of the M13mp18 DNA (Yanisch-Perron et al., 1985), beginning at the priming site, is given:

5´GTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGCCAAGCTTGCATG
CCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCGTAATC
ATGGTCATAGCTGTTTCC-3´.

Fig. 2. Left: Construction of the pAS terminator-probe vector. The *gfp* reporter cassette was inserted into the *Hind*III site (H) of the pLS1 plasmid. This cassette contains a multiple cloning site (MCS), translation initiation signals optimized for prokaryotes (SD) and a promoter-less *gfp* allele (Miller and Lindow, 1997). Plasmid pSA (control plasmid) carries the *gfp* reporter cassette inserted in the opposite orientation. Right: *gfp* gene expression in plasmid-harbouring cells. *S. pneumoniae* carrying plasmid pAS (black square) or pSA (white square). *E.*

faecalis carrying plasmid pAS (black circle) or pSA (white circle). The graph is the mean of three experiments.

Fig. 3. Palindromic sequences at the terminator regions analyzed in this work. Arrows indicate nucleotide sequences corresponding to potential RNA hairpin structures. Complementary bases of the hairpin structures are shown in bold.

Fig. 4. Main sequence elements at the promoter regions analyzed in this work. The –35 and –10 hexamers are indicated with brackets. The position of the extended –10 element (5´-TRTG-3´ motif) is shown. Conserved nucleotides are indicated in bold.

Fig. 5. Fucose-induction of *gfp* expression in pneumococcal cells carrying plasmid pAST-*PfcsK*. Cells were grown in media containing 0.3% sucrose and the indicated amount of fucose to an OD_{650} = 0.6. Cells harbouring plasmid pAST-o*PfcsK* were used as control. (A) Western blot analysis using antibodies against GFP. Total proteins from cell extracts were separated by SDS-PAGE (14% polyacrylamide). Pre-stained proteins (Invitrogen) were run in the same gel as molecular weight markers (not shown). (B) Intensity of fluorescence in cultures (400 µl) of pneumococcal cells carrying the pAST-*PfcsK* plasmid. The intensity of fluorescence in cultures of cells carrying the pAST-o*PfcsK* plasmid was 43.22 ± 2.30 in the absence of fucose and 41.15 ± 1.81 in the presence of 1% fucose. In each case, three independent cultures were analyzed.

Table 1
Oligonucleotides used in this study.

Name	Sequence (5´ to 3´)
Primer extension	
INTc	CTCGCCTGTTCTCTCATCAAC
pAS construction	
F-*gfp*	CCATGATTACGCC**AAGCTT**GG (*Hind*III)
R-*gfp*	CCCCCGGGTACC**AAGCTT**GAATTCC (*Hind*III)
Terminator regions	
F-*T1T2rrnB*	CGATGGTAGTGTGGG**GTCGAC**CCATGCGAGA (*Sal*I)
R-*T1T2rrnB*	TGACGACAGGAAGAGTTT**GTCGAC**ACGCAA (*Sal*I)
F-*TpolA*	ACTAAGATGCTGTTACAA**GTCGAC**GATGAA (*Sal*I)
R-*TpolA*	CTTGGATTGATAAATT**GTCGAC**TCATAG (*Sal*I)
F-*TrsiV*	GGATGCCCCCAGTGATG**GTCGAC**CCTTC (*Sal*I)
R-*TrsiV*	TGTTATGCTTAATTCTA**GTCGAC**GCTTCT (*Sal*I)
Promoter regions	
F-*PsulA*	ACATGATTGTTAATG**GGATCC**CTTTCTG (*Bam*HI)
R-*PsulA*	TCACTCCCTCAA**GGATCC**TCATCATAT (*Bam*HI)
F-*Pung*	CGAAAGAGGTAGTA**GGATCC**TTAATGAT (*Bam*HI)
R-*Pung*	TGTTCCATAGCCGACT**GGATCC**TTTTTACTGCCTC (*Bam*HI)
F-*PuppS*	AAAATTTTA**GAGCTC**GGCAGATAC (*Sac*I)
R-*PuppS*	CCCTCCATTCCAA**GAGCTC**TATCTTAATT (*Sac*I)
F-*P2493*	AATTAAATAG**GAGCTC**GGATGTTAAATATC (*Sac*I)
R-*P2493*	CACGATTGAACAAG**GAGCTC**AAATACATTATT (*Sac*I)
F-*P2962*	CAATTAAGGCCCT**GGATCC**AGCAAAAAGT (*Bam*HI)
R-*P2962*	CGCGCCCATTCACC**GGATCC**CTTAATC (*Bam*HI)
F-*PfcsK*	TATTATAGCACAA**TCTAGA**GGAATTTG (*Xba*I)
R-*PfcsK*	CCATTTTTCTTCTC**TCTAGA**TCCTTGATTAAC (*Xba*I)

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Table 2

Use of plasmid pAS as a terminator-probe vector.

Inserted fragment	Intensity of fluorescence [a]	
	S. pneumoniae 708	*E. faecalis* JH2-2
None	139.54 ± 13.22	259.54 ± 24.49
T1T2rrnB	46.08 ± 3.99	58.92 ± 2.64
T2T1rrnB	77.70 ± 4.71	176.32 ± 8.59
TpolA	158.90 ± 4.35	85.14 ± 10.94
TrsiV	45.75 ± 2.24	54.78 ± 2.07

[a] The intensity of fluorescence in pneumococcal and enterococcal cells carrying the control plasmid pSA was 46.32 ± 2.24 and 58.08 ± 0.64, respectively. Cells harbouring plasmid were exponentially grown to an OD_{650} = 0.3, as indicated in Materials and Methods. The fluorescence (arbitrary units) corresponds to 0.8 ml of culture. In each case, three independent cultures were analyzed.

Table 3

Use of plasmid pAST as a promoter-probe vector.

Promoter	Intensity of fluorescence [a]	
	S. pneumoniae 708	*E. faecalis* JH2-2
None	46.08 ± 3.99	58.92 ± 2.64
PsulA	247.67 ± 16.97	137.68 ± 0.90
Pung	472.23 ± 27.82	118.82 ± 10.53
PuppS	99.03 ± 6.49	102.62 ± 1.44
P2493	166.19 ± 4.23	533.11 ± 18.86
P2962	70.84 ± 2.98	167.43 ± 9.75

[a] The intensity of fluorescence (arbitrary units) corresponds to 0.8 ml of culture (OD_{650} = 0.3). In each case, three independent cultures were analyzed.

1

2

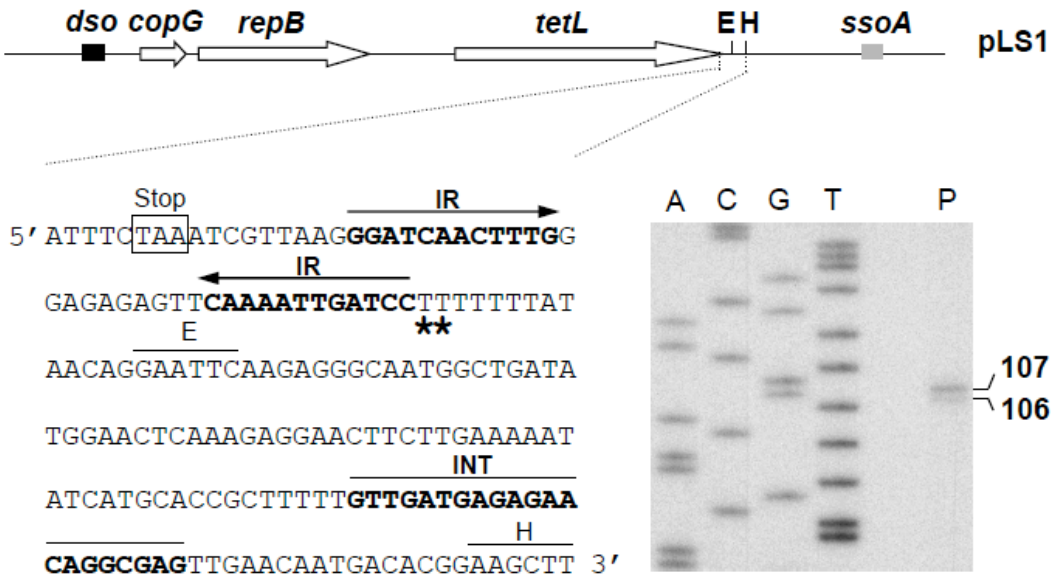

Figure 1 **Ruiz-Cruz *et al.***

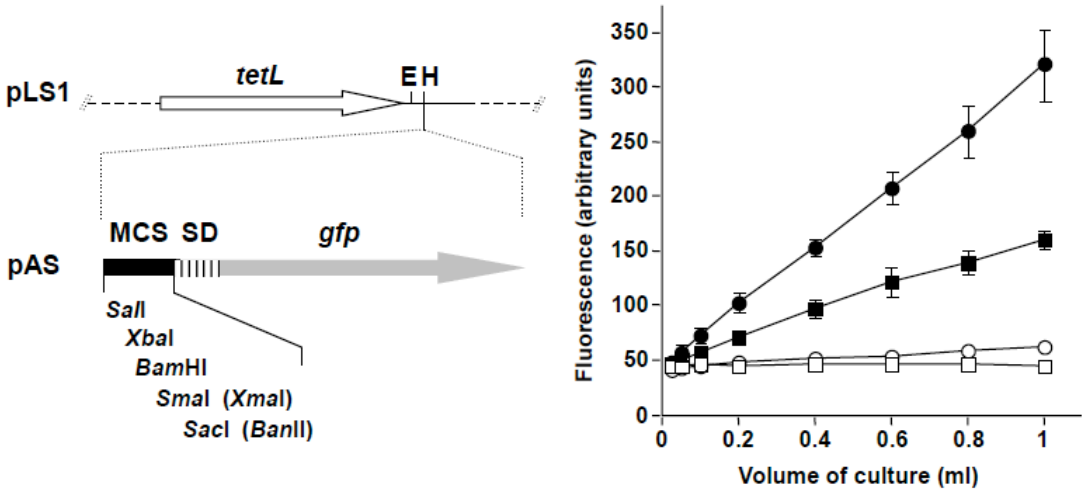

Figure 2 **Ruiz-Cruz *et al.***

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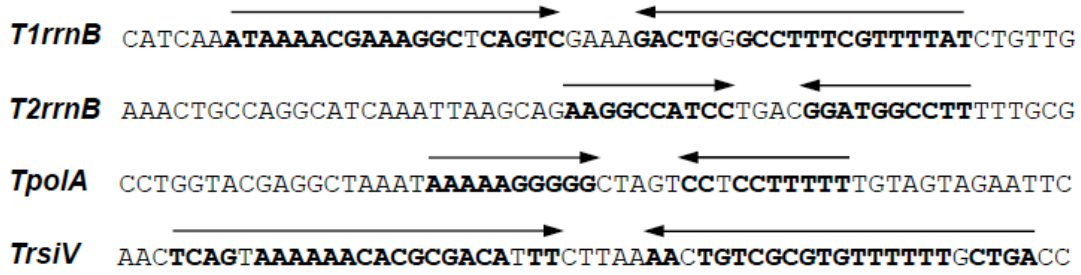


Figure 3 **Ruiz-Cruz *et al.***

1

2

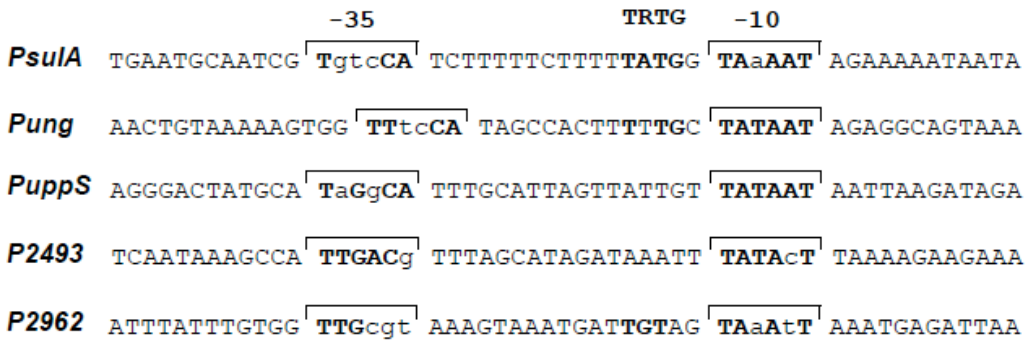
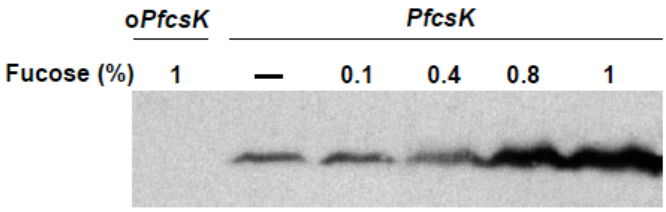


Figure 4 **Ruiz-Cruz *et al.***

1

2

A



B

Fucose (%)	Fluorescence (arbitrary units)
—	64.66 ± 5.95
0.1	91.96 ± 5.51
0.2	129.14 ± 5.22
0.4	205.45 ± 10.17
0.6	287.75 ± 10.88
0.8	322.12 ± 50.97
1	339.41 ± 52.24

Figure 5 **Ruiz-Cruz *et al.***

1